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Kaempferol inhibits VEGF expression and *in vitro* angiogenesis through a novel ERK-NFκB-cMyc-p21 pathway

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Abstract

Kaempferol has been reported to reduce the risk of ovarian cancer, but the mechanism is not completely understood. In this study, we tend to expand our understanding on how kaempferol regulates VEGF expression and angiogenesis in ovarian cancer cells. We timed VEGF secretion, and studied *in-vitro* angiogenesis by kaempferol treatment. Gene expression was examined by qRT-PCR, ELISA, Western Blotting, or luciferase assay, and pathways were examined by manipulating genetic components with plasmid or siRNA transfection. It was found that kaempferol time-dependently inhibited VEGF secretion, and suppressed *in-vitro* angiogenesis. Kaempferol down-regulated ERK phosphorylation as well as NFκB and cMyc expression, but promoted p21 expression. Examination of relationship between these genes suggested a novel ERK-NFκB-cMyc-p21-VEGF pathway, which accounts for kaempferol's angioprevention effects in ovarian cancer cells. This data supplements our comprehension of the mechanisms behind kaempferol's biological influence in ovarian cancer cells, and better characterized kaempferol toward chemoprevention.

Keywords

kaempferol; VEGF; Angiogenesis; ERK

1. Introduction

The death rate of ovarian cancer has not seen any remarkable changes for over 5 decades in the US (Jemal et al., 2010), and the most recent studies failed to find better biomarkers, or marker panels, for screening and early diagnosis than the existing CA125 (Cramer et al., 2011; Zhu et al., 2011), which remains unsatisfying in terms of sensitivity and specificity for early detection of ovarian cancers (Bast et al., 1998; Winstead, 2011). This situation is challenging but also provides plenty opportunities for chemoprevention of early-stage

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ovarian cancers in women. While the search for better biomarkers continues, the strategy of chemoprevention with dietary compounds appears attractive for the vast majority of healthy women who are more, or less, at a risk of developing ovarian cancer.

Study on thousands of female American registered nurses suggested that women with a diet rich in kaempferol have a significantly and surprisingly lowered risk for developing ovarian cancers (Gates et al., 2007), although the mechanism is unexplained. Kaempferol is a natural flavonoid, widely distributed in a variety of fruits and vegetables including strawberry, broccoli, tea, and others (Häkkinen et al., 1999; Justesen et al., 1998; Kuhlmann et al., 2009). Before application of kaempferol for chemoprevention in women, however, its working mechanisms need to be delineated and its effects need to be confirmed. Our earlier study found that kaempferol inhibits *in vivo* angiogenesis induced by ovarian cancer cells, and the pathways involve regulation on HIF1- α and ERR- α gene expressions (Luo et al., 2009).

Anti-angiogenesis is probably the most feasible strategy for current chemoprevention of ovarian cancers, given the fact that these early-stage tiny tumors cannot be successfully diagnosed but their continued development desperately depends on the establishment of a new blood vessel network (Bertl et al., 2006; Carmeliet et al., 2000). Tumor-free adults, on the other hand, have virtually zero need for angiogenesis in normal situations (Fotsis et al., 1993; Glade-Bender et al., 2003). Thus our findings about kaempferol's anti-angiogenesis effects (Luo et al., 2009) become more relevant in this context. Nevertheless, the previously investigated pathways for kaempferol are not believed to be exclusive, and we tend to expand our understandings of the mechanisms behind kaempferol's biological effects. In this study, we further investigate kaempferol and angiogenesis in ovarian cancer cells through novel pathways, and hope to provide new insight into the mechanisms and potentials for chemoprevention with kaempferol.

2. Material and methods

2.1. Cell Culture and Treatment

Ovarian cancer cell lines OVCAR-3 and A2780/CP70 were provided by Dr. Jiang at West Virginia University, and A2780/wt was a kind courtesy from Dr. Tew at University of South Carolina. They were maintained in RPMI 1640 medium (Sigma) supplemented with 10% US-qualified fetal bovine serum (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. Kaempferol (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 100 mM and equal amount of DMSO was included in controls for every experiment. Human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (ATCC) and cultured in Vascular Cell Basal Medium (ATCC) supplemented with Endothelial Cell Growth Kit-VEGF (ATCC).

2.2. Cell Proliferation Assay

OVCAR-3 and A2780/CP70 ovarian cancer cells were seeded in 60-mm dishes at 5E5 cells/dish and incubated for 16 hours before treatment with 3 ml medium containing DMSO or 20- μ M kaempferol. At hours 0.5, 6, 12, 24, 30, and 48, culture medium were collected and frozen for VEGF quantification, and cells were trypsinized, stained with trypan blue and counted under a phase contrast microscope with a hemocytometer.

2.3. ELISA for VEGF and p-ERK

For quantification of VEGF₁₆₅ protein, culture medium collected from aforementioned Cell Proliferation Assay were analyzed with a Quantikine Human VEGF Immunoassay Kit (R&D Systems) following the manufacturer's instructions. For quantification of p-ERK,

OVCAR-3 and A2780/CP70 cells were seeded in 60-mm dishes at 1E6 cells/dish and incubated overnight before treatment with various concentrations of kaempferol for 2 hours. Cells were rinsed twice with phosphate buffered saline (PBS), harvested with lysis buffer, and analyzed with a Phospho-ERK1(T202/Y204)/ERK2(T185/Y187) Cell-Based ELISA Kit (R&D Systems). Cell lysates were also assayed for total protein levels using the BCA Protein Assay Kit (Pierce) to adjust p-ERK levels.

2.4. In-Vitro Angiogenesis Assay

A2780/CP70 cells were seeded in 6-well plate at 2E6 cells/well and incubated overnight. Cells were then treated with 2 ml serum-reduced (1% FBS) medium containing 0, 20, 40, or 80 μ M kaempferol for 24 hours. The conditioned medium were collected and store at -80°C for *in vitro* angiogenesis assay with an “*In Vitro* Angiogenesis Assay Kit, Tube Formation” from Trevigen. Basement membrane extract (BME) were plated in cold 96-well plate at 50 μ l/well and incubated at 37°C for 30 minutes. HUVEC were stained with 2- μ M Calcein AM at 37°C for 30 minutes, trypsinized and harvested, mixed with equal volume of conditioned medium, and plated on gelled BME at 2E4 cells/well. After 6-hour incubation at 37°C , tube formation was visualized under a fluorescence microscope (485 nm excitation/ 520 nm emission) and pictures were taken for comparison.

2.5. Construction of Plasmids

The VEGF promoter reporter pGL-StuI was constructed by inserting a 2.65-kb fragment of the human VEGF gene promoter into the pGL2 basic luciferase vector as described previously (Forsythe et al., 1996). Plasmids for GFP-ERK1 (Addgene 14747), PCMV4 p50 (Addgene 21965), cMyc promoter (TBE1/2-wt) (Addgene 16595), pMXs-hc-MYC (Addgene 17220), were obtained from Addgene. Plasmid SR-alpha was used as vector control and beta-galactosidase plasmid served as normalizer to adjust for transfection efficiencies.

2.6. Transient Transfection and Luciferase Assay

OVCAR-3 and A2780/CP70 cells were seeded in 24-well plates at 2E5 cells/well and incubated overnight. Cells were transfected with 1 μ g DNA (0.2 μ g promoter reporter plasmid, 0.2 μ g beta-galactosidase plasmid, 0–0.6 μ g tested plasmid or SR α plasmid) and 4 μ l transfectmine 2000 (Invitrogen) as per instructions for 6 hours. The cells were then treated with, or without, 40 μ M kaemperol for 6 hours, and harvested for luciferase assay with a ONE-Glo Luciferase Assay System (Promega) and beta-galactosidase assay with a beta-galactosidase Enzyme Assay System (Promega) following the manufacturer’s instructions. Levels of luciferase were normalized by beta-galacotosidase to account for transfection efficiencies.

2.7. Western Blot

OVCAR-3, A2780/CP70, and A2780/wt cancer cells were seeded and incubated overnight before treatment with kaempferol and/or plasmid-transfection, as indicated in individual experiments. After a double wash with cold PBS, cells were harvested with M-PER Mammalian Protein Extraction Reagent (Pierce) supplemented with Halt Protease and Phosphatase Inhibitor (Pierce), and total protein levels were assayed with BCA Protein Assay Kit (Pierce). Cell lysates (50 μ g total protein) were separated by SDS-PAGE and blotted onto nitrocellulose membrane with a Mini-Protean 3 System (Bio-Rad). For immunodetection, antibodies against NF κ B, cMyc, and GAPDH (Santa Cruz) were applied and signals visualized with corresponding second antibodies, SuperSignal West Pico substrate, and x-ray films (Pierce). Protein bands were quantitated with NIH ImageJ software and normalized by GAPDH bands for analysis.

2.8 qRT-PCR

The effects of kaempferol or siRNAs on cMyc, p21, and VEGF mRNA levels were determined by quantitative reverse-transcription PCR (qRT-PCR). OVCAR-3 and A2780/CP70 cells were seeded and treated as indicated in individual experiments. Cells were washed with PBS, harvested in TRIzol reagent (Invitrogen) to extract total RNA. RNA samples were quantitated at OD 260/280 and 1 µg RNA was introduced to reverse transcription with AMV Reverse Transcriptase (Promega). cDNA, equivalent to 80 ng total RNA, was amplified by real-time PCR in triplicate with RT² SYBR Green qPCR Master Mix (SuperArray) and a Chromo4 real-time detector coupled to a DNA Engine thermal cycler (Bia-Rad). Primers for GAPDH and cMyc were chosen from the PrimerBank website (<http://pga.mgh.harvard.edu/primerbank/>), and primers for p21 and VEGF were designed from the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are listed in Table 1. The PCR program was set as follow: 95°C 10 min; (95°C 20 sec, 58°C 45 sec, 72°C 20 sec, 77°C 1 sec, read plate) × 50 cycles; 72°C 5 min; 58°C 1 min; melting curve (65 °C –95 °C by 0.5 °C increment). A standard curve for each gene was generated from serial dilutions of PCR products to monitor amplification efficiency and to relatively quantitate mRNA abundance. RNA samples without reverse transcription served as a non-reverse-transcription (–RT) control, and water served as a non-template control (NTC). Arbitrary units of each gene were derived from corresponding standard curves and normalized by GAPDH levels.

2.9. siRNA Transfection

siRNAs for negative control (Invitrogen), as well as siRNAs against cMyc (Invitrogen) and p21 (Ambion) were screened and effective knock-downs were further studied for their effects on other genes. siRNAs (200 nM) were reverse-transfected into OVCAR-3 cells (8,000 cells/well) in 96-well plate with 1.0 µl siPORT NeoFX Transfection Agent (Applied Biosystems) following the manufacturer's instructions. After 48 hours incubation, cell viability was determined with a "CellTiter 96 Aqueous One Solution Cell Proliferation Assay" kit (Promega), and gene knock-downs as well as effects on other genes were analyzed by qRT-PCR. All results were expressed as percentages of negative control siRNA. A total of 4 independent experiments with triplicates each were carried out for statistical analysis.

2.10. Statistical Analysis

Average values of replicates were collected from independent experiments and analyzed by ANOVA and Post-Hoc Test (2-sided Dunnett t) in SPSS 15.0 (SPSS Inc) to test overall differences and to specifically test the differences between individual treatments and control. A p value of less than 0.05 is considered significant.

3. Results and Discussion

3.1. Kaempferol Inhibits Cell Proliferation and VEGF Expression Time-dependently in Ovarian Cancer Cells

Our earlier study demonstrated that kaempferol inhibits cell proliferation and VEGF expression concentration-dependently in ovarian cancer cells (Luo et al., 2009), but also raises a question about the time needed for kaempferol to exert its effects, especially at physiologically more relevant concentrations. Here we further examined the time course of kaempferol treatment at a low concentration (20-µM) in ovarian cancer cells (Figure 1). For both VEGF levels (Figure 1A) and cell number counts (Figure 1B), kaempferol only exhibits its inhibition on or after 24 hours treatment. Normalization of VEGF abundance by cell numbers could bring in more errors because VEGF is secreted into culture medium and

cumulative, while live-cell count is a cross-sectional estimation. Nevertheless, our normalized VEGF levels point out that kaempferol only showed discernable inhibition on, or after, 24 hours treatment in both OVCAR-3 and A2780/CP70 cells (Figure 1C). This data agrees well with our previous findings that even a higher concentration of kaempferol (40- μ M) only inhibits VEGF mRNA expression on or after 16 hours in ovarian cancer cells (Luo et al., 2009), and it is necessary for a lower concentration of kaempferol (20- μ M) to take a longer time to show its inhibition on VEGF expression at protein levels. These experiments demonstrated that kaempferol can be effective in inhibiting cell proliferation and VEGF protein expression in ovarian cancer cells at physiologically more relevant concentrations, although this dietary flavonoid needs longer time to show its effects.

3.2. Kaempferol Inhibits In Vitro Angiogenesis in Ovarian Cancer Cells

Suppressed expression of VEGF by kaempferol in ovarian cancer cells is expected to lead to inhibited angiogenesis due to the role played by VEGF in angiogenesis (Gupta and Zhang, 2005). Although kaempferol has been proven effective in inhibiting *in-vivo* angiogenesis (Luo et al., 2009), we wonder about its direct effect on vascular epithelial cells. As shown in Figure 2, culture medium collected from A2780/CP70 ovarian cancer cells without kaempferol treatment effectively promoted tube formation by HUVEC cells, showing a large network of endothelial tubes. With increasing concentrations of kaempferol treatment, the network was scattered down and HUVEC cells gradually lose their ability to form tube structures. A 40- μ M kaempferol treatment completely destroyed the network structure and HUVEC cells present as mostly individual clumps at 80- μ M kaempferol treatment. Although the 20- μ M kaempferol treatment does not appear remarkably different, this is consistent with our time-course of VEGF expression for A2780/CP70 cells (Figure 1C), where the normalized VEGF abundance at 24-hour treatment is not lower than that of control by 20- μ M kaempferol treatment. However, 24-hour treatment by 20- μ M kaempferol effectively reduced VEGF secretion in OVCAR-3 cells (Figure 1C), probably accounting for its effectiveness in *in vivo* angiogenesis assays (Luo et al., 2009). Here our experiment demonstrated that HUVEC cells do respond discriminately to kaempferol-treated and non-treated cell culture medium, presumably due to distinctly different abundance of VEGF, among other factors, that is secreted by ovarian cancer cells under varying kaempferol concentrations.

3.3. Kaempferol Inhibits ERK Signaling in Regulation of VEGF

Despite an earlier failure in finding any inhibition of p-ERK levels after 24-hour treatment (Luo et al., 2009), concentration-dependent inhibition of ERK phosphorylation was captured at 2-hour kaempferol treatment in both OVCAR-3 and A2780/CP70 ovarian cancer cells (Figure 3A), and a significance was reached at a low concentration of 10 μ M. This is closer to the *in-vivo* concentration of 1.4 μ M (400 ng/ml) kaempferol in blood reported in rats (Chen et al., 2010). To investigate whether ERK signaling is involved in kaempferol's regulation on VEGF, we transfected ovarian cancer cells with ERK1 plasmid and found that kaempferol's inhibition on VEGF transcriptional activation, as reflected by VEGF reporter luminescence, was abolished by forced expression of ERK1 in a concentration-dependent manner, and a significance was achieved in A2780/CP70 cells (Figure 3B). This data suggested that not only kaempferol inhibits ERK phosphorylation and VEGF expression in ovarian cancer cells, but also this inhibition of VEGF is dependent on, at least partly, suppression of ERK signaling. While it is common between kaempferol and apigenin, a well-studied natural dietary flavonoid, for their inhibition of VEGF expression in cancer cells and their implementation of PI3K/Akt pathway (Fang et al., 2005; Liu et al., 2005; Luo et al., 2009), it is worth noting that kaempferol appears quite unique in our study by influencing another signaling molecule, ERK, which is shown completely quiescent in ovarian cancer cells treated with apigenin (Fang et al.,).

3.4. Kaempferol Inhibits NFκB Expression through ERK Signaling Pathway

Due to its wide involvement in multiple pathways and broad regulation targets (Aggarwal, 2004), transcription factor NFκB is investigated in ovarian cancer cells treated with kaempferol. As shown in Figure 4, kaempferol treatment concentration-dependently and significantly reduced NFκB protein levels in all 3 ovarian cancer cells. To learn whether NFκB is downstream of ERK signaling in kaempferol-treated ovarian cancer cells, A2780/CP70 and OVCAR-3 cells were transfected with ERK1 plasmid, treated with kaempferol, and analyzed for NFκB abundance. As shown in Figure 5, NFκB levels, though reduced by kaempferol treatment, climbed up by forced expression of ERK1 plasmid in a concentration-dependent and significant manner in both ovarian cancer cell lines. The fact that kaempferol inhibits ERK signaling and NFκB expression, but the latter is contingent upon the former suggests that transcription factor NFκB is down-regulated through ERK signaling in kaempferol-treated ovarian cancer cells.

3.5. Kaempferol Inhibits VEGF Expression through Regulating cMyc Gene

Since onco-gene cMyc has been reported to play a role in angiogenesis (Bertl et al., 2006; Kokai et al., 2009), it becomes interesting to see whether cMyc is involved in kaempferol's regulation on VEGF and whether cMyc is activated by NFκB transcription factor in ovarian cancer cells. Our study found that cMyc is down-regulated by kaempferol treatment concentration-dependently and significantly at both mRNA and protein levels (Figure 6). Furthermore suppression of cMyc expression is a prerequisite for kaempferol's regulation on VEGF in ovarian cancer cells, as transfected plasmid cMyc concentration-dependently reversed kaempferol's inhibitory effects, and a significance is reached in A2780/CP70 cells (Figure 7A). In addition to the finding that kaempferol regulates VEGF through down-regulating cMyc in ovarian cancer cells, our study further revealed that cMyc is a target of NFκB in kaempferol-treated ovarian cancer cells, and forced expression of NFκB1 will abolish kaempferol's inhibitory effects on cMyc in a concentration-dependent manner (Figure 7B).

3.6. Kaempferol Up-regulates p21 in Ovarian Cancer Cells

P21 (CDKN1A) is a known target to cMyc (Jung and Hermeking, 2009) and consequently is expected to be involved in kaempferol's regulation on VEGF in ovarian cancer cells. Real-time PCR analysis revealed that even at very low concentrations (5–10 μM), kaempferol already significantly promoted P21 expression in both OVCAR-3 and A2780/CP70 cells (Figure 8A). As the blood concentration of kaempferol can be 1.4 μM (400 ng/ml) in rats (Chen et al., 2010), it is possible that a 5-μM kaempferol in vivo is reachable and will enhance p21 expression in ovarian cancer tissues. To confirm p21's role in kaempferol's regulation on VEGF, OVCAR-3 cells were knocked-down for cMyc or p21 by siRNAs, and analyzed for cMyc, p21, and VEGF mRNA levels by real-time PCR. As shown in Figure 8B, siRNA cMyc Duplex 1 successfully knocked down cMyc mRNA expression, and as a consequence, p21 mRNA levels were significantly up-regulated, demonstrating that cMyc reverse-regulates p21 in ovarian cancer cells. Next, when siRNA CDKN1A 242474 successfully knocked down p21 mRNA expression, VEGF mRNA levels were stimulated significantly, suggesting p21's role in regulation of VEGF in ovarian cancer cells; cMyc gene is, however, unaffected by p21 siRNA, confirming in this pathway that cMyc is upstream of p21 and regulates p21, but not vice versa. Cell viability is slightly affected (at about 80% of control) by both siRNA transfections. Overall, this data implicates the pathway that cMyc regulates p21, and p21 regulates VEGF expression in ovarian cancer cells.

4. Conclusions

Our study supplemented our existing knowledge about kaempferol and angioprevention in ovarian cancers (Luo et al., 2009) with a new mechanism of ERK-NF κ B-cMyc-p21-VEGF pathway (Figure 9), and added to better sounded explanation to kaempferol's impressive effects in chemoprevention of ovarian cancers found in epidemiological studies (Gates et al., 2007). Of course, as an *in-vitro* model, cell culture cannot take Absorption, Distribution, Metabolism and Excretion (ADME) of kaempferol into consideration, and more often than not, the concentrations applied above are not reachable in *in-vivo* models. Nevertheless, our *in-vitro* experiments help to delineate the pathways involved, and call for more relevant *in-vivo* studies. As a widely distributed dietary flavonoid, kaempferol has had its effects shown in humans and its working mechanisms better understood in cell models. Both lines of evidence suggest it a potential chemoprevention agent. Further studies in animal models and human trials are warranted to promote this natural compound toward chemoprevention of ovarian cancer cells.

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Research Highlights

- In this study we analyzed kaempferol's inhibitory effect on VEGF expression and angiogenesis in ovarian cancer cells.
- We identified ERK-NFκB-cMyc-p21 pathway as a novel mechanism to explain kaempferol's effects.
- This new pathway supplements our understanding of the mechanisms and paves the way to future chemoprevention.

Figure 1.

Kaempferol time-dependently inhibits cell proliferation and VEGF secretion ovarian cancer cells. OVCAR-3 cells and A2780/CP70 ovarian cancer cells were seeded in 60-mm dishes and treated with DMSO or 20- μ M kaempferol for different duration. At hours 0.5, 6, 12, 24, 30, and 48, culture medium were collected and frozen for VEGF analysis, and cells were trypsinized, stained with trypan blue and counted under microscope with a hemocytometer. **A:** Total VEGF secreted into culture medium as determined by ELISA. **B:** Cell numbers counted with hemacytometer. **C:** Secreted VEGF levels were normalized by corresponding cell numbers. Data represents Means $\pm$ SE from 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ as compared to control

Figure 2.

Kaempferol inhibits *in vitro* angiogenesis in ovarian cancer cells. A2780/CP70 ovarian cancer cells were seeded in 6-well plate and treated with kaempferol in serum-reduced (1% FBS) medium for 24 hours. The conditioned medium were collected for *in vitro* angiogenesis assay: 50 μ l BME was pre-dispensed in chilled 96-well plate and incubated at 37 °C to gel. HUVEC cells (3E4) were stained with calcein, mixed with equal volume of conditioned medium, plated onto BME gel, and incubated for 6 hours. Pictures were taken under a fluorescence microscope

Figure 3.

ERK signaling is involved in kaempferol's regulation on VEGF. **A:** Kaempferol inhibits ERK phosphorylation. OVCAR-3 and A2780/CP70 cells were seeded into 60-mm dishes and treated with kaempferol for 2 hours. Cells were harvested and quantitated for phospho-ERK1 (dually phosphorylated at T202/Y204) and phospho-ERK2 (dually phosphorylated at T185/Y187) by ELISA. Cell lysates were also determined for total protein levels by BCA assay to normalize ELISA results. Data represents means and SE from 4 independent experiments. * $p < 0.05$; ** $p < 0.01$ as compared to control. **B:** ERK mediates kaempferol's inhibitory effect on VEGF transcriptional activation. OVCAR-3 and A2780/CP70 cells were seeded in 24-well plate and incubated overnight. Cells were transfected with plasmids of VEGF reporter, beta-galactosidase, ERK1, and SR- α (as vehicle) by transfectmine2000 for 6 hours. The cells were then treated with, or without, 40- μ M kaempferol for 6 hours, and harvested for luciferase assay and beta-galactosidase assay. Data represents means $\pm$ SE from 3 independent experiments. ## $p < 0.01$ as compared to control; ** $p < 0.01$ as compared to kaempferol-treated control

Figure 4.

Kaempferol inhibits NFκB1 expression in ovarian cancer cells. A2780/CP70, A2780/wt, and OVCAR-3 ovarian cancer cells were seeded in 60-mm dishes, incubated overnight, and treated with kaempferol for 24 hours. Then cells were harvested, proteins separated by SDS-PAGE, and detected for NFκB1 (p105) and GAPDH proteins in western blots. Typical blot bands were shown at top. Protein bands were quantified with NIH ImageJ software and NFκB1 protein levels were normalized by corresponding GAPDH levels. Data represents means $\pm$ SE from 2 independent experiments. *p<0.05 as compared to control; **p<0.01 as compared to control.

Figure 5. ERK mediates kaempferol's inhibitory effects on NFκB1 in ovarian cancer cells. A2780/CP70 and OVCAR-3 ovarian cancer cells were seeded in 6-well plates at 1.5E6 cells/well and incubated overnight. Cells in each well were transfected with 0–4 μg ERK1 plasmid using SR-alpha plasmid as vehicle control and 16 μl lipofectamine2000 for 4–6 hours, followed by treatment with 0- or 40-μM kaempferol for 24 hours. Cells were harvested with M-PER Mammalian Protein Extraction Reagent supplemented with Halt Protease and Phosphatase Inhibitor, separated by SDS-PAGE, and detected for NFκB1 and GAPDH proteins by Western blot. Protein bands were quantitated by NIH ImageJ software and NFκB1 levels were normalized by GAPDH levels. Data represents means ± SE from 3–4 independent experiments. * $p < 0.05$ as compared to control. ** $p < 0.01$ as compared to control

Figure 6.

Kaempferol inhibits cMyc expression in ovarian cancer cells. **A:** Ovarian cancer cells (5E5) were seeded in 60-mm dishes and treated with kaempferol for 24 hours. Total RNA was extracted with TRIzol Reagent, reverse-transcribed with AMV reverse transcriptase, and quantitated by SYBR Green-based real-time PCR. cMyc mRNA levels were normalized by GAPDH abundance and expressed as percentages of control. Data represents means $\pm$ SE from 3 independent experiments. **B:** Ovarian cancer cells (5E5) were seeded in 60-mm dishes and treated with kaempferol for 24 hours. Cells were harvested with M-PER Mammalian Protein Extraction Reagent, and cell lysates were separated by SDS-PAGE and blotted into nitrocellulose membrane for immunodetection. Chemiluminescent signals were captured by x-ray film and quantitated by imaging software. cMyc protein levels were normalized by GAPDH and expressed as percentages of control. Typical blots were shown and data represents means $\pm$ SE from 3 independent experiments. * $p < 0.05$ as compared to control. ** $p < 0.01$ as compared to control

Figure 7.

Kaempferol inhibits VEGF expression through NFκB-cMyc pathway. **A:** cMyc Mediates Kaempferol's Inhibitory Effect on VEGF Transcriptional Activation in Ovarian Cancer Cells. OVCAR-3 and A2780/CP70 cells were seeded in 24-well plate and incubated overnight. Cells were transfected with VEGF reporter, beta-galactosidase, hc-Myc plasmid, and SRα plasmids (as vehicle) with transfectmine2000 for 6 hours. The cells were then treated with, or without, kaempferol for 6 hours, and harvested for luciferase assay and beta-galactosidase assay. Data represents means $\pm$ SE from 3 independent experiments. ## $p < 0.01$ as compared to control; * $p < 0.05$ as compared to kaempferol-treated control. **B:** NFκB1 mediates kaempferol's inhibitory effects on cMyc expression in ovarian cancer cells. Ovarian cancer cells were seeded in a 24-well plate at 2×10^5 cells in 500 μ l growth medium per well and incubated overnight. Cells were transfected with 0.2 μ g cMyc reporter, 0.2 μ g beta-galactosidase, 0–0.6 μ g NFκB1 plasmid, and SR-alpha plasmids (as vehicle) by 4 μ l transfectmine2000 for 6 hours. The cells were then treated with or without 40 μ M kaempferol for 8 hours, and harvested for luciferase assay and beta-galactosidase assay. Data represents means $\pm$ SE from 2–3 independent experiments. # $p < 0.05$ as compared to control; ## $p < 0.01$ as compared to control; * $p < 0.05$ as compared to kaempferol-treated control.

Figure 8.

Kaempferol inhibits VEGF expression through cMyc-p21 pathway. **A:** Kaempferol promotes p21 (CDKN1A) expression at mRNA level in ovarian cancer cells. Ovarian cancer cells (5E5) were seeded in 60-mm dishes, incubated 16 hours, and treated with kaempferol for 24 hours. Total RNA was extracted with TRIzol Reagent, reverse-transcribed with AMV reverse transcriptase, and quantitated by SYBR Green-based real-time PCR. CDKN1A mRNA levels were normalized by GAPDH abundance and expressed as percentages of control. Data represents means $\pm$ SE from 3 independent experiments. * $p < 0.05$ as compared to control. ** $p < 0.01$ as compared to control. **B:** P21(CDKN1A) mediates cMyc's effect on VEGF expression in ovarian cancer cells. OVCAR-3 cells (8E3/well) were reverse-transfected with 200-nM siRNA by NeoFX, incubated 48 hours, analyzed for cell viability by MTS-base assay, and studied for gene expression at mRNA level by qRT-PCR. Gene expression levels were normalized by GAPDH and expressed as percentages of control. Data represents means $\pm$ SE from 4 independent experiments. * $p < 0.05$ as compared to control. ** $p < 0.01$ as compared to control.

Figure 9. Known and newly proposed pathways for kaempferol's effect on angiogenesis in ovarian cancer cells

Table 1

Primer Sequences for qRT-PCR

Gene	GenBank Accession	Primers	Amplicon size	Source
VEGF	NM_001025368	AAG GAG GAG GGC AGA ATC AT ATC TGC ATG GTG ATG TTG GA	226	Primer3
GAPDH	NM_002046	CAT GAG AAG TAT GAC AAC AGC CT AGT CCT TCC ACG ATA CCA AAG T	113	PrimerBank
cMyc	NM_002467	TCG GAA GGA CTA TCC TGC TG GTG TGT TCG CCT CTT GAC ATT	133	PrimerBank
p21	NM_000389	TTA GCA GCG GAA CAA GGA GT AGC CGA GAG AAA ACA GTC CA	226	Primer3